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13. ABSTRACT *(Maximum 200 words)*

This report covers studies of regenerating peripheral nerves and the effect such nerves exert on regenerative growth of the tissue innervated. The iron-transport protein transferrin is an absolute requirement for cell proliferation and is abundant in peripheral nerves. The hypothesis investigated here is that transferrin is delivered axonally and is involved in the nerve-dependent cell proliferation which characterizes repair in avascular tissues. Amphibian (axolotl) limb regeneration is a well-characterized model system for nerve-dependent reparative growth and was used here in experiments testing the hypothesis. Results include demonstrations that transferrin is present in both axons and Schwann cells of peripheral nerves, that the concentration of this factor increases greatly during regeneration, that transferrin is transported distally in regenerating axons at the expected rate for fast axonal transport in amphibians, and is released at the growing tips of such axons. Previous work has shown that when nerves to regenerating axolotl limbs are transected the concentration of transferrin in the distal limb tissue declines rapidly and limb regeneration stops. These results strongly support the hypothesis that neural transferrin is important in nerve-dependent growth during vertebrate limb regeneration. Studies of both transferrin binding and expression of the transferrin gene in cells of axolotl peripheral nerve indicate that both uptake and synthesis of this factor occur in the regenerating nerve. These results have important implications for understanding the trophic effect of nerves in tissue repair.

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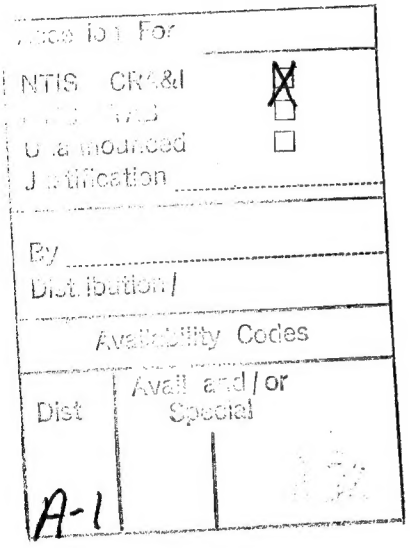

INTRODUCTION

That peripheral nerves exert a "trophic" influence on certain processes of tissue repair or regeneration has long been recognized (rev. Drachman, 1974). Reparative processes in which growth clearly depends on an adequate nerve supply include healing of corneal epithelial abrasions (Beuerman and Schimmelpfenning, 1980), replacement of taste buds (Zalewski, 1974), and regeneration of amputated limbs in urodele amphibians (Carlone and Mescher, 1985). Of these processes amphibian limb regeneration has been studied by far the most extensively in attempts to characterize the trophic activity. The molecular basis for the neural effect on tissue repair remains unknown but various factors have been suggested (Brockes, 1984; Carlone and Mescher, 1985). We are examining the role of the iron-transport factor transferrin, a protein abundant in nervous tissue which is required for maintenance and proliferation of cells (Mescher and Munaim, 1988).

The iron requirement for survival and growth of animal cells is based on the need for this atom in the prosthetic groups of many important enzymes, including enzymes necessary for aerobic respiration and for DNA synthesis (Aisen, 1982). Certain specialized cells also need iron as a cofactor in proteins related specifically to the cells' function, such as hemoglobin or enzymes involved in synthesis of collagen or production of various neurotransmitters. The principal mechanism by which cells of vertebrate tissues obtain iron under physiological conditions is receptor-mediated endocytosis of transferrin (Aisen and Listowsky, 1980; Morgan, 1981). Work with several types of cells has established the basic pattern of iron-transferrin uptake: transferrin complexed to its receptor is internalized via clathrin-coated vesicles, the iron atoms are transferred to other cytoplasmic proteins, and the receptor-apotransferrin complex is returned to the cell surface where transferrin is

released to bind iron again for another delivery cycle (Bomford and Munro, 1985; Huebers and Finch, 1987).

Increased numbers of transferrin receptors appear on the cell surface in response to mitogenic growth factors and such receptors are very abundant on all proliferating cells (May and Cuatrecasas, 1985; Huebers and Finch, 1987). Studies with synchronized cells have shown that the increased expression of transferrin receptors coincides with the onset of DNA synthesis (Seligman, 1983; Bomford and Munro, 1985). The importance of transferrin for DNA replication is apparently due to the iron requirement of ribonucleotide reductase, the rate-limiting enzyme for DNA synthesis (Eriksson et al., 1984).

Additional evidence for the importance of transferrin in cell growth comes from observations that proliferation *in vitro* can be blocked by addition to the medium of monoclonal antibodies to the transferrin receptor or by chelation of iron from the medium (Seligman, 1983). Moreover, normal cells cultured in defined media will only grow if transferrin is present (Barnes and Sato, 1980). *In vivo*, adverse effects on growth caused by severe iron deficiency have been shown both clinically (Morgan, 1981) and experimentally (Kochanowski and Sherman, 1982).

Transferrin is primarily synthesized and secreted in the liver and delivered to cells throughout the body in the plasma. However, local synthesis of transferrin occurs in tissues where cells do not have direct access to plasma proteins. In the seminiferous tubules of the testis, for example, developing germ cells bind transferrin released from Sertoli cells, which both synthesize this protein (Skinner and Griswold, 1980) and take it up selectively from the interstitial fluid (Morales and Clermont, 1986). In rodent brain transferrin has been shown to be synthesized by both the choroid plexus (Dickson et al., 1985) and oligodendrocytes

(Bloch et al., 1985), apparently to meet the iron requirements of neurons which also lack direct access to transferrin from plasma. Transcytosis of transferrin has also be demonstrated across epithelial cells of the blood-testis barrier (Morales and Clermont, 1986) and the blood-brain barrier (Fishman et al., 1987).

Peripheral nerves are particularly rich in transferrin (Markelonis et al., 1982; Meek and Adamson, 1985), but the cells responsible for uptake and/or synthesis of the factor in this tissue are not known. Recent work has shown increased uptake of transferrin and iron by Schwann cells of rat sciatic nerve during post-traumatic nerve regeneration (Raivich et al., 1991). The importance of iron in neural tissue probably involves its role in enzymes for aerobic respiration and for neurotransmitter synthesis (Wrigglesworth and Baum, 1988) and neuronal growth seems to require increased levels of iron delivery. Studies on the synthesis and possible significance of transferrin in peripheral and central nervous tissue have been extensively reviewed elsewhere (Mescher and Munaim, 1988).

In attempts to characterize the nerve's trophic agent required for growth of vertebrate limb regeneration blastemas, extracts of amphibian brain have been used with various *in vivo* and *in vitro* bioassays (Singer, 1974; Carlone and Mescher, 1985). Measuring cell proliferation in cultured regeneration blastemas, initial studies with heterologous transferrin in this laboratory showed stimulation of DNA synthesis and mitotic activity, with a biphasic dose-response curve and an optimal response similar to those obtained with brain extract (Mescher and Munaim, 1984). The mean mitotic index of blastemas cultured with an optimal concentration of transferrin was not significantly different from that of blastemas of the same age *in vivo* (with normal innervation). We subsequently showed through the use of an iron chelator that the growth-promoting effect of brain extract was dependent on its

content of iron, which suggested that transferrin may be involved in the stimulatory effects of neural extracts in this bioassay (Munaim and Mescher, 1986).

For *in vivo* investigations on the role of neural transferrin during limb regeneration, we purified transferrin from the salamander *Ambystoma mexicanum* and produced a variety of monoclonal and polyclonal antibodies against this factor. We have used these antibodies in two ways. First, we have developed a noncompetitive, solid-phase enzyme-linked immunosorbent assay (ELISA) for *Ambystoma* transferrin that is capable of measuring as little as 1 ng of transferrin per ml of tissue extract.

We have found that interruption of the nerve supply to mid-bud stage forelimb regenerates (six days after amputation) of larval *Ambystoma* reduces the transferrin content of distal blastema tissues by more than 50%. Similar reductions were found at various times after axotomy, ranging from six days to one day before distal tissues were harvested for assay. Care was taken not to disrupt the vascular supply to the limbs during the denervation procedure. Since denervation inhibits cell proliferation in limb stumps and transferrin uptake is greater in growing than in nongrowing cells, reduction of the transferrin content in distal limb tissues by denervation could be an indirect effect in which blocked growth causes decreased uptake of transferrin by cells. As a control therefore, larval salamander forelimbs were X-irradiated (2000 rads) unilaterally and amputated bilaterally one day later. Six days after amputation the shielded limbs had mid-bud stage blastemas, but the X-irradiated limbs showed no sign of regeneration. However, transferrin concentrations in distal tissues were similar in both X-irradiated and regenerating limbs, indicating that transferrin content was not dependent on the state of growth in the tissue.

We used the same assay for transferrin content in recent work demonstrating that this factor is transported in regenerating *Ambystoma* peripheral nerve (Kiffmeyer et al., 1991). This study will be discussed very briefly here; experimental details, statistical analysis and complete discussion of the data are included in the publication cited and in the Final Report of the previous USAMRDC contract. Using a standard procedure for the study of axonal transport *in vivo* (Bisby, 1982), sciatic nerves were exposed in regenerating limbs of adult salamanders and ligated at a distal level in the stylopodia. One day later the animals were killed and entire sciatic nerves, including associated dorsal root ganglia, were removed and cut into segments. Each segment was homogenized and its transferrin content determined by the ELISA. Consistently, a 2 to 3-fold accumulation of transferrin was present proximal to the ligature, suggesting axonal transport of the factor distally. Control experiments, in which axonal transport is disrupted by local application of colchicine to the neurons, clearly indicated that transferrin accumulation due to axonal transport rather than to edema or vascular constriction. Such controls are particularly important when testing for axonal transport of a protein abundant in plasma.

Immunocytochemical investigations were performed on both sections and teased preparations of *Ambystoma* brachial nerves. The results with individual myelinated fibers in teased nerves indicate that transferrin is present in both axons and Schwann cells (Kiffmeyer et al., 1991). Longitudinal sections of nerve and dorsal root ganglia also show transferrin localization in neurons and Schwann cells (Tomusk and Mescher, 1988). These studies showed further that the protein is particularly abundant in the perineurium and perineuronal glial cells of the ganglia, structures which constitute major features of the blood-tissue barrier in the peripheral nervous system. Electron microscopy reveals that the

flattened processes of perineurial fibroblasts, which are present as multiple lamellae surrounding each fascicle of nerve fibers, have numerous vesicles and pits opening to both sides of the cell. Such ultrastructural characteristics are typical of flattened cells involved in "transcytosis", i.e., transcellular transport and exocytosis of endocytosized material. An important question regarding the possible role of transferrin in the trophic effect of nerves is whether this factor is released distally from regenerating nerves. Released from axons at a significant concentration, transferrin would be expected to exert a permissive influence locally on cells if their supply of iron-transferrin from plasma or other sources was not adequate to support growth. As reviewed in detail elsewhere (Mescher and Munaim, 1988), studies of limb regeneration indicate that the early, nerve-dependent blastema is essentially avascular and is characterized by extensive extracellular proteolysis. Such microenvironmental conditions would reduce the supply of transferrin available to blastemal cells from capillaries. Therefore neural transferrin may provide an explanation for the nerve-dependence of cell proliferation in this system: upon its release from axons, transferrin would bind iron available locally and be taken up by cycling blastema cells.

As stated in the proposal for this study, *our major hypothesis is that neurons of peripheral nerves accumulate transferrin from the surrounding glial cells, transport it distally along axons, and release it from growth cones.* As described briefly above, we have obtained good evidence for axonal transport *in vivo*. We are now investigating further the physiological importance of transferrin in peripheral nerves by studying axonal transport and release of the factor, which requires development of a suitable *in vitro* model, and by determining the mechanism by which transferrin accumulates in peripheral nerves. The specific purpose of these experiments is to answer several questions, all of which are of

fundamental importance to understanding the physiological and developmental significance of transferrin in peripheral nerves. The major questions to be answered, as indicated in the proposal, include the following:

(1) Can axonal transport of transferrin be demonstrated in regenerating peripheral nerves in organ culture?

(2) Is transferrin released at regenerating nerve endings (growth cones)?

(3) Is transferrin taken up by neuronal cell bodies and/or axons across the blood-nerve barrier?

(4) Do neurons or other cells of peripheral nerves synthesize transferrin?

Several different methods have been used to answer these questions. Axonal transport and distal release of transferrin must be investigated in nerve organ culture in order to detect and quantify the secretion of transferrin from growth cones. To undertake this we have adapted methods of sciatic nerve organ using serum-free culture medium in multicompartment chambers, a method others have used to show axonal release of various unknown proteins. Combining this approach with the ELISA for quantitative measurement of axolotl transferrin developed with the previous USAMRDC support has allowed us for the first time to demonstrate axonal transport and release of a specific protein from distal ends of regenerating axons. As discussed in a recent review (Mescher, 1992), this work has considerable significance not only for the role of nerves in promoting regenerative growth as studied here, but also for other systems in which growth and maintenance of a structure depends on peripheral nerves.

The experimental method for the binding study used light microscope autoradiographic analysis after binding of radiolabelled transferrin. Studies of transferrin

synthesis employed cDNA probes for axolotl transferrin mRNA generated by the polymerase chain reaction (PCR) used, in both Northern analyses and *in situ* hybridization. As discussed in the next section, the use of PCR-generated probes represents a change from the method originally proposed.

BODY

Organ culture studies designed to answer the two questions regarding the transport and release of transferrin from regenerating peripheral nerves were completed successfully using the methods proposed and the results have been published (Kiffmeyer et al., 1991; Kiffmeyer, 1991; Mescher and Kiffmeyer, 1992). This work confirmed the *in vivo* results described above and produced the first demonstration that transferrin is released in physiologically significant quantities from distal regenerating ends of axons. These results are consistent with and extend reports from other investigators showing increased uptake of iron-transferrin (Raivich et al., 1991) and other plasma proteins (Boyles et al., 1990) in regenerating rat sciatic nerves. Moreover, *they demonstrate for the first time axonal transport and distal release of a "trophic" factor with well-understood and important significance as a requirement for cell growth*. Such a demonstration has never been accomplished *in vivo* in this or any other nerve-dependent developing system and the results have major significance for the field of regeneration.

To determine whether transported proteins are secreted distally at growth cones of regenerating axons an *in vitro* approach using organ culture of injured, regenerating peripheral nerve was necessary. Axonal growth into connective and mesenchymal tissue during regeneration of amputated amphibian limbs precludes direct study of distally released factors *in vivo*. However axonal regeneration following crush injury to a nerve occurs within the morphologically intact perineurium rather than in non-neural tissue. Dissection of such regenerating nerves with ganglia attached and perineurium intact can be followed by their transfer to organ culture. This approach allows isolation of material secreted distally from the growth cones of regenerating axons. This *in vitro* method of studying distally secreted

proteins was developed initially by others using frog sciatic nerves (e.g., Tedeschi and Wilson, 1987) and was adapted in our laboratory for use with axolotl nerves since this urodele species rather than frogs is of particular interest with regard to the question of limb regeneration.

Axonal regeneration was stimulated bilaterally by standard crush injuries to adult axolotl sciatic nerves at the level of the knee. Four weeks later each injured nerve was dissected from the limb, including dorsal root ganglia, the site of the crush injury, and at least 1 cm of nerve distal to the injury. Each nerve was cleaned of extraneous connective tissue and blood vessels and placed in a multicompartment culture slide (Labtek) inside a 10-cm culture dish (Fig, 1). All chambers and the dish were filled with serum-free Liebovitz L-15 medium (GIBCo), diluted to 80% for amphibian osmolarity and containing GIBCo antibiotic-antimycotic. For each nerve, ganglia were placed in one chamber and the distal portion containing the regenerating growth cones was placed in a second chamber. Nerve tissue draped across the chamber walls was covered with petroleum jelly to prevent desiccation. The intermediate region of nerve was bathed in medium of the culture dish between the two chambers, thereby preventing passive diffusion of material between chambers along the nerve. After different periods of culture (typically 24 hr) in a humidified chamber at 22°C, medium from the proximal and distal chambers was assayed for transferrin in triplicate using the ELISA described above.

Results with six cultured nerves are shown in Figure 2. In every case there was greater release of transferrin from the regenerating nerve distally than from the ganglia, so that the mean transferrin concentration in the distal chamber was twice that in the chamber containing the ganglia. Cultures maintained for three days with media sampled every twelve

hours revealed that release was linear through approximately the first day, then leveled off (Fig. 3). The decline in transferrin release after 24 hours may be due to depletion of the protein from nerves or to a reduced rate of axonal transport in the cultured nerve.

Although *in vitro* preparations of injured, regenerating frog sciatic nerves have been used by others to investigate fast axonal transport and release of material during neuronal growth (Hines and Garwood, 1977; Tedeschi and Wilson, 1987; Synder, 1988), it is particularly important with a plasma protein to show that release is associated with axoplasmic transport and not simply passive diffusion from interstitial tissue space. In our preparations a greater volume of tissue was present in the proximal ganglionic chamber, suggesting that the two-fold higher concentration of transferrin found in the distal compartment was not due to diffusion from the tissue. To test this point further, colchicine was added to the compartment containing the ganglia in order to block axoplasmic transport. In every case this was found to prevent accumulation of transferrin in the medium of the distal chamber surrounding the growth cones (Fig. 4), indicating that this accumulation is dependent on anterograde transport in the axons.

The average transferrin concentration in the distal medium after 24 hrs of culture exceeded 200 ng/ml (Kiffmeyer et al., 1991). Released locally from regenerating axons within the microenvironment of a blastema or other site of nerve injury *in vivo*, this amount of transferrin would represent a significant contribution to the micromolar concentrations required for optimal proliferation of blastema cells (Albert and Boilly, 1988) and mammalian cells (Barnes and Sato, 1980).

Our finding that transferrin is fast-transported and released in regenerating axons is consistent with data from other groups studying axonal transport of secretory proteins.

Using two-dimensional polyacrylamide gel electrophoresis to analyze material released *in vitro* from regenerating sciatic nerves of frogs, Tedeschi and Wilson (1987) found that approximately five newly synthesized, fast-transported polypeptides were secreted in significantly greater amounts. The spots were not identified, but prominent among them was one for a glycoprotein with a size and isoelectric point similar to that of transferrin. In a study using similar techniques, Stone and Hammerschlag (1987) identified transferrin among the fast-transported proteins in frog sciatic nerve by testing various antisera on Western blots of the two dimensional polyacrylamide gels.

To determine whether transferrin is taken up by nerves of injured and uninjured peripheral nerve, we attempted to use methods of light microscopy and autoradiography. Dorsal root ganglia and attached lengths of brachial or sciatic nerve, as well as segments of spinal cord at the level of the regenerating motor nerves, were sectioned at 10 microns thickness on a cryostat. Slides containing these sections were treated with I^{125}-transferrin, washed, and exposed to photographic emulsion in standard procedures for light microscope autoradiography to demonstrate the locations of the cells binding transferrin.

Despite several attempts we were not successful in obtaining useful data in these experiments because of high background binding which led to excessive and nonspecific labeling on the autoradiograph. Binding of radiolabeled transferrin appears to be present along the length of the peripheral nerve, which is consistent with the results of Raivich et al. (1991) for rat sciatic nerve following crush injury, but it is not yet possible to ascertain which cells of the nerve are involved. The binding study should be repeated with appropriate dilutions of radiolabeled transferrin in order the more precisely localize autoradiographically which cells of the nerve sheath are involved in transferrin uptake.

The final area we are investigating involves the question of transferrin **synthesis** within peripheral nerves. To date work in this area has utilized molecular methods and a modification of the ELISA developed earlier. These methods will be described here along with the major results that have obtained.

PolyA+ RNA was isolated from axolotl sciatic nerve, dorsal root ganglia, and spinal cord using messenger affinity paper (mAP, Amersham) using a procedure we have recently published (Sun et al., 1993). Briefly, the tissue was lysed in 50 ul of GT solution (4 M guanidium isothiocyanate, 25 mM sodium citrate, pH 7.0, and 0.5% Sarcosyl, with 2-mercaptoethanol to a final concentration of 0.1 M, and 1/10 volume of 2 M sodium acetate pH 4.0). Squares of the mAP filters measuring 1.5 mm by 1.5 mm were placed on sterile paper towels and saturated with 0.5 M NaCl. The lysed sample was carefully loaded onto the filter 1-2 ul drops at a time so that the sample was drawn through the filter and did not flow off the sides. NaCl (0.5 M) was then passed through the filter in the same manner and the filter was washed twice by placement in a micrifuge tube containing 0.5 ml of 0.5 M NaCl and vortexing. Finally the filter was washed twice in the same manner with 0.5 ml of 70% ethanol and stored in 70% at -20° C. To use the purified mRNA, the mAP filter was dried in a vacuum concentrator, transferred to a new tube, and 12 ul RNAse-free water was added. The tube was heated at 72° C for 5 min to elute the poly-A+ RNA, then chilled on ice and used for reverse transcription.

The reverse transcription reaction mixture was 20 ul in volume and contained: 1X RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.5 mM each dGTP, dATP, dTTP, and dCTP, 5 uM random hexamers, 1 unit Rnase Block II (Stratagene) per 20 ul reaction mixture, 200 units Moloney murine leukemia virus reverse

transcriptase (GIBCO BRL) and 4 ul of poly-A+ RNA isolated as described above. The reaction mixture was incubated at 23° C for 10 min, at 37° for 60 min, then heated at 99° for 5 min.

Oligonucleotide primers for PCR were designed using the published sequence for the cDNA of *Xenopus* transferrin (Moskaitis et al., 1990). The sense and antisense primers were each 25 nucleotides in length, were based on highly conserved regions in the cDNA encoding the 5' domain of the protein, and the primer pair spanned 254 bases in exons 4, 5, and 6. The sequence of the sense primer, including an Eco R1 site at the 5' end, was 5'AGAATTCGGCAAGACTGCTGGATGG 3'. The sequence of the antisense primer, including a 5' Bam H1 site, was 5'CGGATCCCAAACACTTGAATGCACC 3'.

PCR was conducted in a total volume of 50 ul containing: 1X PCR buffer (500 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 0.1 mM each dGTP, dTTP, dATP, and dCTP, 4 ul of RT reaction, 1 unit Taq polymerase, 0.15 uM of the sense and antisense oligonucleotide primers. The reaction was run for 35 cycles at 60° C for 30 sec, 72°C for 30 sec, and 95°C for 30 sec. After PCR, 8 ul of the reaction mixture was analyzed on a 2% agarose gel and stained with ethidium bromide to determine the size of the cDNA reaction product(s). It should be noted that the PCR approach, as indicated in previous quarterly reports, is much less labor intensive than the method originally proposed, which involves construction and screening of cDNA libraries.

Results of a typical RT-PCR using poly-A+ RNA from liver and from normal and regenerating nervous tissues is shown in Figure 5. With RNA from all tissues (liver, spinal cord, dorsal root ganglia, and sciatic nerve) an expected product approximately 254 bp in size was present. Excess cDNA was present in some lanes and smaller bands were present in

some preparations. Although not quantitative, PCR clearly indicates the presence of transferrin mRNA comparable to that of liver in both normal and regenerating tissue from spinal cord, dorsal root ganglia, and sciatic nerve.

Products of three separate PCR preparations were cloned using the TA cloning kit (Invitrogen) and sequenced directly off the plasmid with the Sequenase kit (United States Biochemicals), both according to the manufacturers' recommendations. The sequence obtained is shown in Figure 6. The nucleotide sequence is 89% homologous and the amino acid sequence is 82% homologous to the corresponding region of the *Xenopus* transferrin cDNA (Moskaitis et al., 1990).

To characterize further the transferrin mRNA from axolotl nervous tissue, Northern analysis of RNA was performed by standard methods (Sambrook et al., 1989) using a probe synthesized by RT-PCR as described above, substituting ^{32}P-dCTP in the reaction mixture. Results (Fig. 7) show the presence in RNA preparations from both liver and brain of a single transferrin mRNA, approximately 2.0 Kb in size, which is comparable to transferrin mRNA in other species.

That the mRNA for transferrin is translated and the protein synthesized in peripheral nerves is indicated by preliminary experiments in which distal, nonganglionated portions of axolotl brachial nerves were cultured for 12 hrs in medium containing ^{35}S-methionine to label proteins synthesized by cells of the nerve, then homogenized and processed in the ELISA for axolotl transferrin described previously (Kiffmeyer et al., 1991). With the use of radiolabeled methionine, the newly synthesized portion of transferrin bound to antibodies in the ELISA could be quantified by scintillation counting. Preliminary results with this approach indicate that cells of peripheral nerves do synthesize transferrin (not

shown). This result is consistent with our data from RT-PCR and if confirmed, it will be the first demonstration that cells of the peripheral nervous system synthesize this plasma protein. The site of transferrin synthesis in the distal regions of peripheral nerves and whether the neurons themselves also make the protein will be determined when the *in situ* hybridizations studies are completed. Since oligodendrocytes are responsible for much transferrin synthesis in the brain, one would expect Schwann cells of peripheral nerves to show transferrin synthesis, but resident macrophages may also be involved.

The evidence we have obtained on the uptake, transport, and release of transferrin in regenerating amphibian sciatic nerves, as well as the results suggesting that transferrin synthesis occurs in such nerves, is consistent with the view that this protein may be one of the long-sought factors secreted from axons supporting growth of blastema cells. This hypothesis was also examined using *in vitro* systems developed to examine the effect of neurons or neural extracts on blastema cell proliferation. Results in such studies (reviewed previously, Mescher and Munaim, 1988) strongly suggest that transferrin is one of the factors in nerve extracts responsible for the growth-promoting effect of such extracts in these cultures. Moreover, transferrin alone can stimulate blastema cell proliferation *in vitro* as effectively as neural extract or serum (Mescher and Munaim, 1984; Albert and Boilly, 1988) and can at least temporarily maintain a significant level of mitotic activity when administered locally in denervated blastemas (Mescher and Kiffmeyer, 1992).

Studies on regenerating forelimbs of larval axolotls in the nerve-dependent period have recently shown that removal of axons in the blastema by transection of the brachial nerves lowers the transferrin concentration by 50% in the distal blastema tissue (Kiffmeyer et al., 1991). This reduction is rapid, occurring within one day of denervation, and is related

directly to the loss of axons rather than to the decreased proliferative activity, as indicated by normal levels of transferrin in limb stumps where proliferation was inhibited by X-irradiation. Transferrin levels in the blastema did not decline further, even five days after denervation, suggesting an alternate source for the protein such as the capillaries in more proximal regions of the limb. Specific concentrations of transferrin in the microenvironments of blastema cells after nerve withdrawal cannot be determined and the levels assayed in these experiments cannot therefore be precisely correlated with the transferrin concentrations needed for blastema cell proliferation *in vitro*. However, the magnitude of the axotomy-related loss of transferrin from the blastema supports the hypothesis that axons supplement the amount of this factor available from other sources to the extent that normal levels of blastema cell proliferation may depend on the neural supply of transferrin until ingrowth of capillaries.

Finally, the accumulation of transferrin in axons and neuronal cell bodies of injured nerves is especially significant in light of reports that nerves regenerating after an earlier injury have greater trophic activity for blastema cells in both *in vitro* (Boilly and Albert, 1988) and *in vivo* (Maier et al., 1984) bioassays. Such observations strengthen the idea that a factor mediating the trophic influence of axons on growth of neighboring cells is also important for regeneration of the axons themselves.

CONCLUSIONS

Using a combination of *in vivo* and *in vitro* approaches with reagents generated and assays developed during the previous period of USAMRDC support, we have demonstrated several points highly significant to the question of the trophic influence of nerves on growth of the regenerating amphibian limb. In the earlier study we showed that the concentration of transferrin in injured sciatic nerves of adult axolotls increases up to twenty-fold during the process of regeneration and that this transferrin is contained in both axons and Schwann cells of the sciatic nerve (Kiffmeyer et al., 1991). transferrin appears to be taken up across the perineurium of these nerves (Tomusk and Mescher, 1988), as has also been demonstrated in rat peripheral nerve (Raivich et al., 1991).

Importantly, we have shown that transferrin is transported by fast axonal transport in sciatic nerves (Kiffmeyer et al., 1991). This represented the first demonstration of axonal transport of an important plasma protein (Mescher, 1992). Axonal regeneration following nerve injury affects the quantity of transferrin transported, but not the rate of transport (Kiffmeyer et al., 1991).

With support from the present grant we have extended these findings and strengthened the hypothesis that neural transferrin is important for the trophic influence of peripheral nerves. We have now shown in cultures of injured sciatic nerve that transferrin is released at the distal ends of regenerating axons in a physiologically significant quantity (Mescher and Kiffmeyer, 1992). Moreover, distal release of transferrin sciatic nerves continues for at least 24 hours, then levels off (Mescher and Kiffmeyer, 1992). Both *in vivo* and *in vitro*, transferrin transport and release depends on microtubule-based axonal

mechanisms, consistent with the view that normal fast axonal transport of transferrin-containing vesicles is involved.

These results strongly support the hypothesis that transferrin is a trophic factor by which nerves support limb regeneration. Without reviewing the literature on amphibian limb regeneration here, it should be noted that these results are entirely consistent with all previous work leading to characterization of the putative trophic factor(s) of nerves. Because of this work transferrin is currently accepted as the most likely candidate for this long-sought factor (Stocum, 1995), although more than one such factor may actually be involved in nerve's trophic effect.

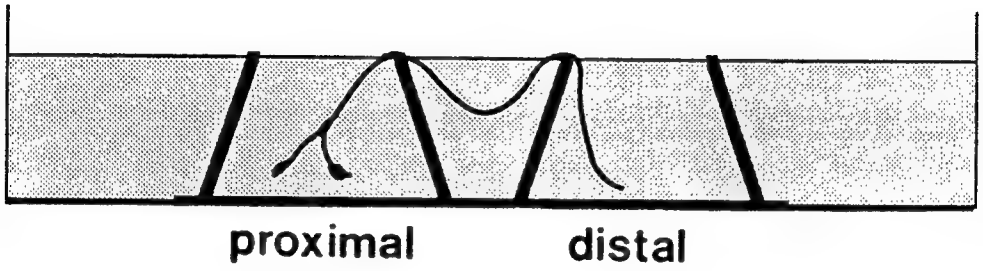

Figure 1. Multichamber system for investigating axonal transport and release of proteins in sciatic nerves *in vitro*. Shown are two chambers on a Lab-Tek slide inside a culture dish. Both chambers and the dish are filled with serum-free medium. Ganglia and the proximal part of the nerve are placed in one chamber and distal portions of the nerve, including the site of axonal regeneration, are placed in the other chamber. The intervening part of the nerve is covered by medium in the dish. At various times of culture a sample of medium is removed from each chamber are tested for its transferrin content by ELISA.

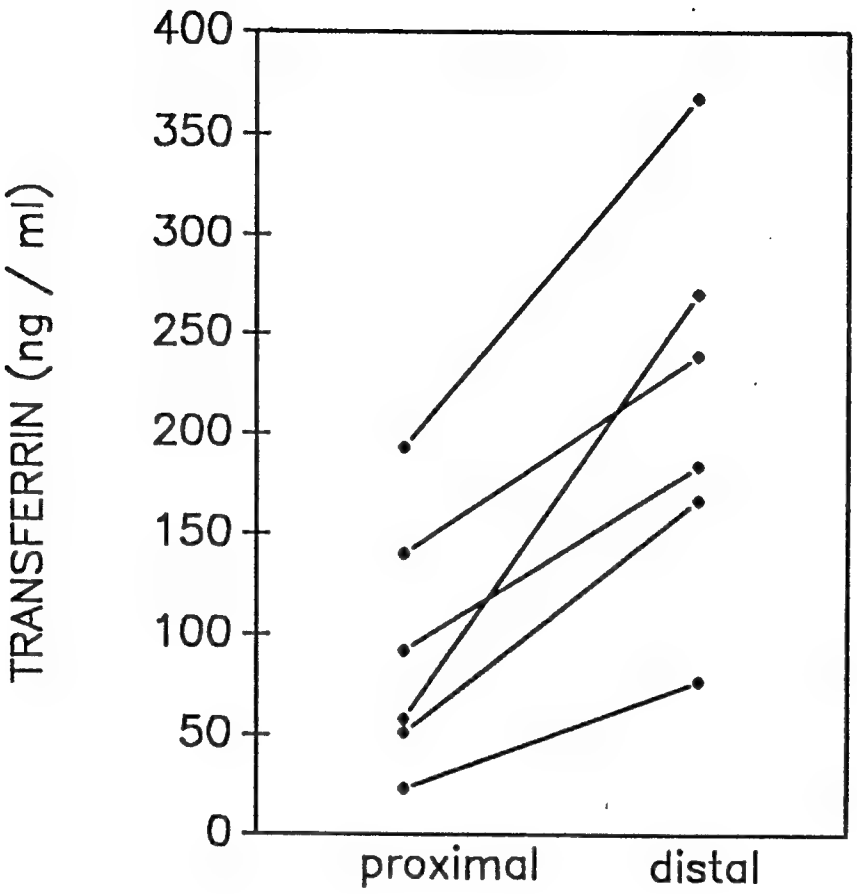

Figure 2. Transferrin concentrations in medium of culture chambers containing proximal and distal regions of regenerating adult axolotl sciatic nerves maintained in organ culture for 24 hours as described in Methods and Materials. Each line represents the results with one nerve. The average concentration in the distal chambers, which contained the regenerating ends of axons injured by crush 4 weeks earlier, was approximately twice that in the proximal chambers containing the dorsal root ganglia.

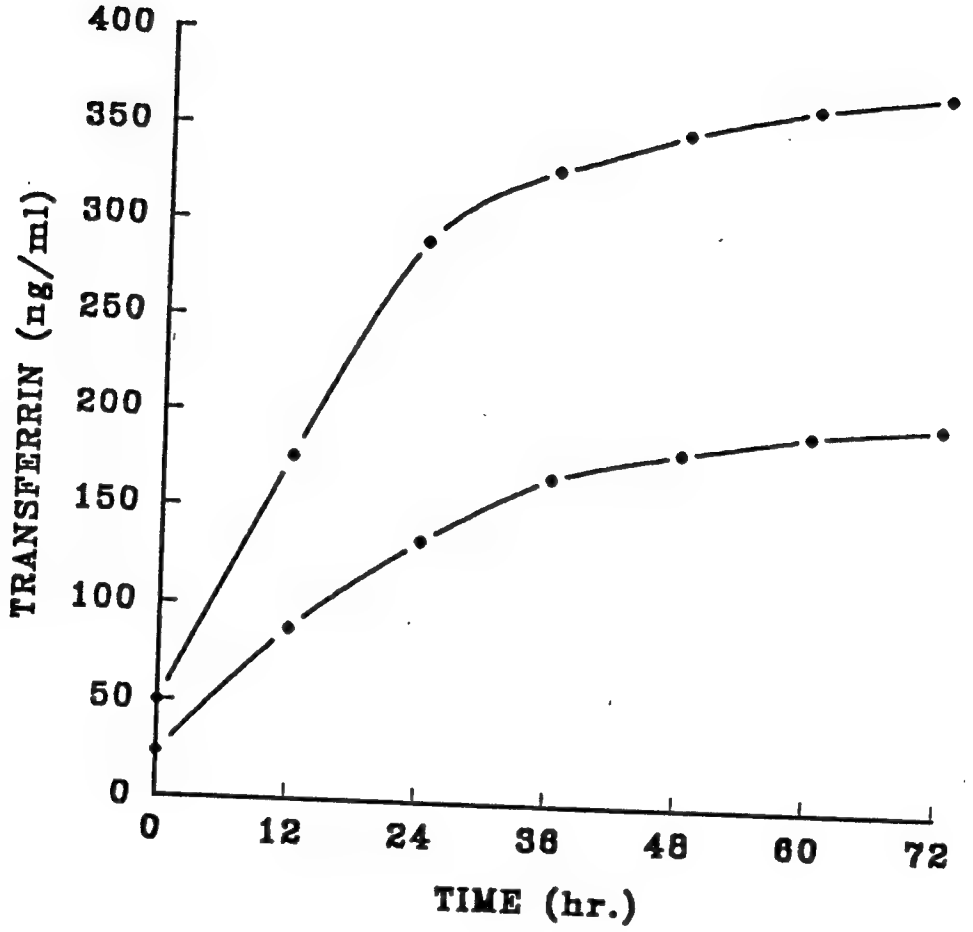

Figure 3. Transferrin concentrations in distal chambers of two representative regenerating adult axolotl sciatic nerves after various periods of culture. Transferrin is released maximally during the first 24-36 hours followed by a gradual decline in the rate of release.

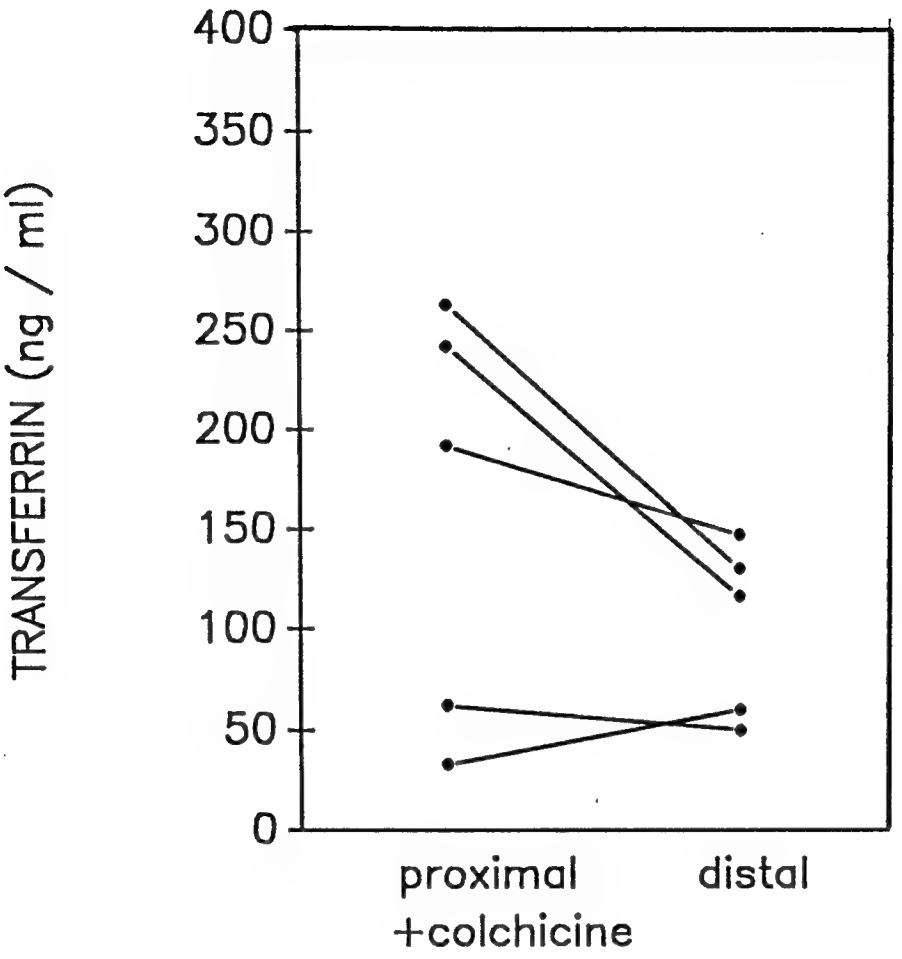

Figure 4. Transferrin concentrations in medium of culture chambers containing proximal and distal regions of regenerating adult axolotl sciatic nerves like those of Fig. 2, but with the addition of colchicine to medium of the proximal chamber. Colchicine in the medium surrounding the ganglia prevented release and accumulation of transferrin in the distal chamber, indicating the importance of axonal transport for this accumulation.

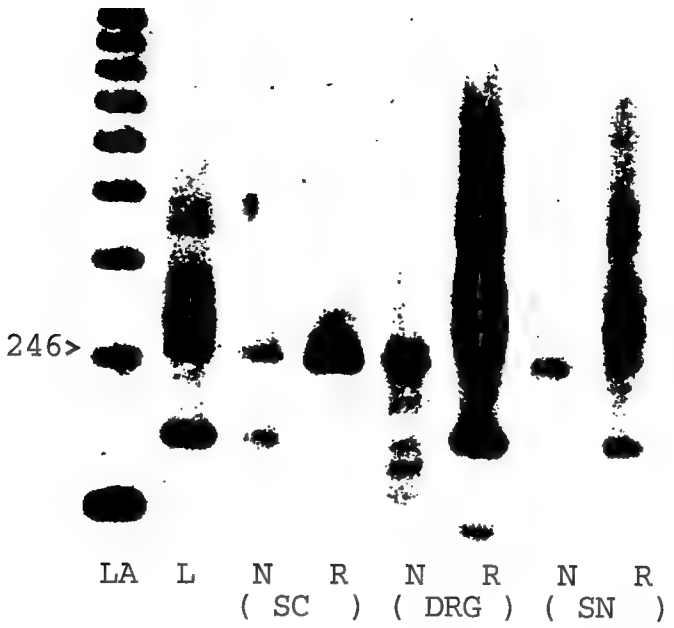

Figure 5. Agarose gel showing 246 bp products of transferrin mRNA reverse transcription - polymerase chain reaction (RT-PCR) with poly-A+ RNA from axolotl liver (L) and from nonregenerating (N) and regenerating (R) nervous tissue from spinal cord (SC), dorsal root ganglia (DRG), and sciatic nerve (SN). At left is a 123 bp ladder (LA). The band indicating transferrin mRNA is present in each sample, although it is obscured here with excess cDNA in samples L, R DRG, and R SN. This result clearly indicates that cells of the axolotl spinal cord, DRG, and peripheral nerves contain mRNA for transferrin.

```
AGTATCATCATTGGACTACTCCTGGAGAAGAAGCTGTTGTCGTGGGGAGGGCCAGATACAGAA
 S  I  I  I  G  L  L  L  E  K  K  L  L  S  W  G  G  P  D  T  E
AGCCTGGAAAAACGCGGTTTCAAGATTCTTCAAAGCCGACTGTGTGCCCGGAGCCAAAGAACCT
 S  W  K  N  A  V  S  R  F  F  K  A  D  C  V  P  G  A  K  E  P
AACTTGTGCCAGCAGTGTGCCGGAAAGAAGGAGCATAAGTGCTCACGCTCTAACAACGAACCT
 N  L  C  Q  Q  C  A  G  K  K  E  H  K  C  S  R  S  N  N  E  P
TACTACAACTATGCT
 Y  Y  N  Y  A
```

Figure 6. Nucleotide and deduced amino acid sequences of the axolotl transferrin cDNA produced by RT-PCR.

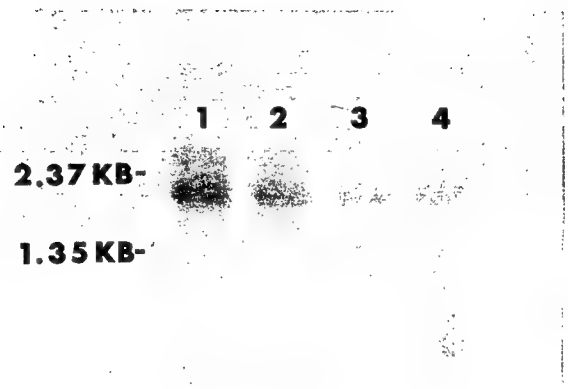

Figure 7. Northern blot showing a single band of approximately 2.0 Kb in RNA from liver and sciatic nerve. Liver RNA was run at 20, 10, and 5 ug (in lanes 1, 2, and 3 respectively). Brain RNA was run at 4 ug (lane 4). This result suggests the presence of a single mRNA for transferrin in these tissues with a size comparable to that of transferrin mRNA in other species.

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APPENDIX

Full-length publications of work supported by DAMD17-91-Z-1002:

Kiffmeyer, W.R. and A.L. Mescher (1991). Axonal transport and release of transferrin in nerves of regenerating amphibian limbs. Developmental Biology **147**, 392-402.

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